

Granzyme A Is an Interleukin 1 β -converting Enzyme

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Summary

Apoptosis is critically dependent on the presence of the *ced-3* gene in *Caenorhabditis elegans*, which encodes a protein homologous to the mammalian interleukin (IL)-1 β -converting enzyme (ICE). Overexpression of ICE or *ced-3* promotes apoptosis. Cytotoxic T lymphocyte-mediated rapid apoptosis is induced by the proteases granzyme A and B. ICE and granzyme B share the rare substrate site of aspartic acid, after which amino acid cleavage of precursor IL-1 β (pIL-1 β) occurs. Here we show that granzyme A, but not granzyme B, converts pIL-1 β to its 17-kD mature form. Major cleavage occurs at Arg₁₂₀, four amino acids downstream of the authentic processing site, Asp₁₁₆. IL-1 β generated by granzyme A is biologically active. When pIL-1 β processing is monitored in lipopolysaccharide-activated macrophage target cells attacked by cytotoxic T lymphocytes, intracellular conversion precedes lysis. Prior granzyme inactivation blocks this processing. We conclude that the apoptosis-inducing granzyme A and ICE share at least one downstream target substrate, i.e., pIL-1 β . This suggests that lymphocytes, by means of their own converting enzyme, could initiate a local inflammatory response independent of the presence of ICE.

IL-1 β -converting enzyme (ICE) is a cytoplasmic cysteine protease characterized from cells of monocytic origin based on its ability to process the 31-kD inactive precursor of IL-1 β (pIL-1 β) to the 17-kD proinflammatory cytokine IL-1 β (1, 2). ICE is a member of a novel class of cysteine proteases with a unique (p20/p10)₂ homodimeric structure (3, 4), and it displays a rare sequence cleavage preference, i.e., after aspartic acids (5, 6). Activation of human pIL-1 β occurs at the Asp₁₁₆-Ala₁₁₇ bond.

Besides their role in the activation of pIL-1 β , ICE and its structural homologue, Nedd2 (7, 8), appear to be involved in the regulation of programmed cell death, or apoptosis. Overexpression of ICE or Nedd2 in rat fibroblasts or neurons induces cell death (7, 9). The CrmA protein, a viral serpin inhibitor of ICE, has been shown to prevent apoptotic cell death in neurons in vitro (10). Moreover, the expression of *ced-3*, a *Caenorhabditis elegans* protein belonging to the ICE family, is essential for programmed cell death to occur during worm development (11).

The only other mammalian protease with aspartase activity described until now is granzyme B, a serine protease expressed in granules of activated cytotoxic lymphocytes and NK cells (12). Along with granzyme A, a protease with trypsinlike specificity, the pore-forming lytic perforin, and other con-

stituents of the cytoplasmic granules, granzyme B is secreted into the intercellular space between killer and target cell upon specific cell-cell interaction (12). The physiological substrate(s) of granzyme A and B remain unknown, but the analysis of granzyme B (-/-) mice points to a crucial role in the control of apoptosis (13). DNA degradation in target cells lysed by lymphocytes deficient in granzyme B is impaired (13). In addition, granzymes A and B induce apoptotic cell death when added together with sublytic doses of perforin (14, 15).

Since these two aspartases occupy key regulatory roles in the induction of the apoptotic cell death program, the question of whether ICE and granzyme B share common substrates arose. We therefore investigated the possibility that the ICE substrate pIL-1 β is processed by lymphocyte granzyme B. In spite of common substrate specificities, we show that only ICE produces active IL-1 β . However, granzyme A was found to be a potent IL-1 β -converting enzyme, suggesting that lymphocytes are able to initiate an inflammatory reaction independent of ICE expression.

Materials and Methods

Reagents and Substrates. Granzyme A substrate BLT (*N*- α -benzyloxycarbonyl-L-Lys-thiobenzyl ester) was purchased from Bachem (Bubendorf, Switzerland), and granzyme B substrate

BAT (Boc-Ala-Ala-Asp-SBzl) was purchased from Enzyme Systems Products (Dublin, CA). The inhibitor for granzyme A (PhNHCO-NHCiTEtOIC, IGA) was a kind gift of Dr. J. Powers (Georgia Institute of Technology, Atlanta, GA), and the ICE inhibitor (Ac-Tyr-Val-Ala-Asp-CHO, YVA) was from the Glaxo Institute for Molecular Biology (Geneva, Switzerland). DCI (3,4-dichloroisocoumarin) was purchased from Boehringer Mannheim (Mannheim, Germany). The polyclonal goat antibody to mouse IL-1 β was raised against purified recombinant protein. The rabbit antibody to human IL-1 β was a kind gift from G. Mazzei (Glaxo Institute for Molecular Biology). The peptide corresponding to the human pIL-1 β sequence Asn₁₁₀ to Asn₁₂₃ was purchased from Bachem.

Purification of Proteases. Granzymes were purified from granules of the B6.1 cytolytic T lymphocyte cell line as described (16). Active human ICE was isolated from the cytosol of the human monocytic cell line THP.1 as previously described (2). ICE was further purified by ion exchange chromatography, sequentially passing the cytosolic fraction over Q-Sepharose fast flow (Pharmacia LKB, Zürich, Switzerland) followed by chromatography on an HR 10 column (Mono-S; Pharmacia LKB). Briefly, the cytosolic fraction was dialyzed against 20 mM Hepes, pH 7.0, 5 mM EDTA, 2 mM dithiothreitol, 1 mM 4-(2-amino ethyl)-benzenesulfonyl fluoride, 0.1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and 15% ethylene glycol (buffer A), and passed over the Q-Sepharose equilibrated with buffer A. The column flow through contained ICE activity and was subsequently chromatographed on Mono-S also equilibrated with buffer A. ICE activity was eluted with a 40-column volume linear gradient from 0–0.5 M KCl in buffer A. This represents a 76-fold purification of ICE activity and sufficiently removed proteases capable of nonspecifically hydrolyzing the ICE fluorescent substrate, Ac-Tyr-Val-Ala-Asp-7-amino-4-methyl coumarin (Bachem). Using this substrate to assay for ICE activity, Mono-S-purified ICE (0.5 mg/ml) had a specific activity of 0.12 nmol AMC min⁻¹ mg⁻¹.

Enzymatic Activity Assays. Granzyme A activity was measured using BLT as a substrate (17). Granzyme B activity was assayed as described for granzyme A, but BLT was replaced by BAT (18). For inhibition studies, the enzymes were preincubated at 20°C for 30 min with the respective inhibitors at the following final concentrations: 170 μ M DCI, 70 μ M IGA, and 2 μ M YVA.

Expression of pIL-1 β . In vitro transcription and translation of human pIL-1 β was carried out with the TNT Coupled Reticulocyte Lysate System (Promega Corp., Madison, WI) according to the manufacturer's instructions. The human pIL-1 β cDNA (Glaxo Research and Development, Greenford, UK), cloned into the pcDNA I Amp expression vector (Invitrogen, NV Leek, The Netherlands) was transcribed using the T7 promoter. For processing, 1 μ l of [³⁵S]methionine-labeled pIL-1 β was incubated with enzyme (0.2 μ g granzyme A, granzyme B, or Mono-S-purified ICE) in a buffer containing 50 mM Tris-HCl, pH 8.0, and 1 mM CaCl₂ for 10 h at 30°C (for digestion with ICE, 0.5 mM dithiothreitol was included). Inhibition of the enzymes was performed as described above. Reaction mixtures were analyzed by SDS-PAGE and subsequent autoradiography. Recombinant bacterial human pIL-1 β was kindly provided by P. Graber (Glaxo Institute for Molecular Biology).

IL-1 Biological Assay. Recombinant human pIL-1 β (200 ng) was incubated with granzyme A (20 ng) during 4 h at 37°C, and IL-1 β activity was assayed as described (19).

Amino Acid Sequencing and Matrix-assisted Laser Desorption Mass Spectroscopy. Granzyme A-processed pIL-1 β was separated by SDS-PAGE, transferred to transblot membranes (Bio-Rad Laboratories,

Zürich, Switzerland) and subjected to amino acid sequence determination using a pulsed liquid phase protein sequencer (477 A; Applied Biosystems, Foster City, CA) on-line phenylthiohydantoin-amino-acid analyzer (120 A; Applied Biosystems).

The peptide corresponding to the pIL-1 β sequence Asn₁₁₀ to Asn₁₂₃ of human pIL-1 β (Bachem, 10 μ g) was incubated with purified granzyme A or B (1 μ g each) at 37°C for 16 h, and the cleavage site was determined by laser desorption mass spectroscopic analysis (20).

Lysis by CTLs. Bone marrow-derived macrophages from BALB/c mice (H2^d) were stimulated with LPS (10 ng/ml) for 3 h, washed, and then added to the *Plasmodium falciparum* circumsporozoite protein peptide/H2^d specific CTL clone PbCSF12 (21). The effector to target cell ratio was 4:1. After 4 h, cells were centrifuged and the cell-associated IL-1 β was determined by Western blot analysis using a goat anti-mouse IL-1 β polyclonal antiserum.

Results

As ICE and the lymphocyte granzymes are both crucial mediators of apoptosis, we investigated whether these proteases recognize and cleave common substrates. Potential cleavage of in vitro-translated human pIL-1 β by granzyme A or B was investigated. As expected, the 31-kD precursor form of IL-1 β was efficiently cleaved by ICE after a 10-h incubation into a fragment corresponding to mature IL-1 β (Fig. 1 A). In spite of the shared substrate specificity of granzyme B and ICE, no cleavage of pIL-1 β was observed with the former enzyme. However, incubation with purified granzyme A generated a fragment similar in size to the authentic 17-kD IL-1 β . Cleavage of pIL-1 β by granzyme A was rapid and already observed after 30 min (data not shown). The granzyme A-induced pIL-1 β conversion was completely blocked by serine protease inhibitor DCI, whereas the ICE inhibitor YVA was inactive (Fig. 1 A). In turn, ICE activity was completely inhibited by YVA at identical concentrations (Fig. 1 A).

Efficient cleavage of pIL-1 β by granzyme A was also observed when recombinant human pIL-1 β produced in bacteria was used as a substrate (Fig. 1 B). Recombinant pIL-1 β gave rise to two bands at \sim 31 kD by Western blot analysis, which were converted to two species of 16–17 kD upon addition of granzyme A. Both processed species showed an identical NH₂ terminus, indicating that the recombinant protein was degraded at its COOH terminus. The conversion was again inhibited with DCI and IGA. Granzyme B had no effect on pIL-1 β .

ICE cleaves between Asp₁₁₆ and Ala₁₁₇ of human pIL-1 β to release the active cytokine. Sequence analysis of granzyme A-converted pIL-1 β revealed Ser₁₂₁ at its NH₂ terminus, indicating that pIL-1 β was cleaved after Arg₁₂₀, in perfect agreement with its thrombinlike activity (Fig. 2 A). Three minor contaminants were found with NH₂ termini corresponding to Leu₇₄, Ser₁₃₃, and Ala₁₄₄. The Arg₁₂₀-Ser₁₂₁ cleavage site was also obtained when a synthetic peptide covering the ICE pIL-1 β cleavage site (NEAYVHDAP-VRSLN) was offered to granzyme A (Fig. 2 B). In spite of comparable esterolytic activity on their respective substrates (BLT and BAT), in no case was cleavage detected after the authentic Asp₁₁₆ by granzyme B.

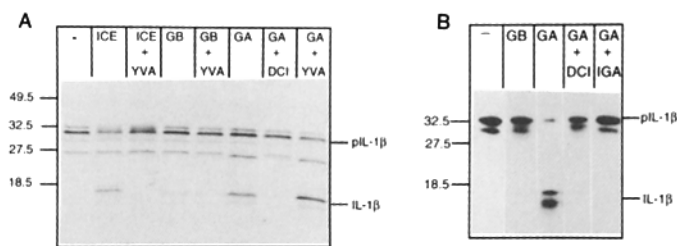


Figure 1. (A) Cleavage of human pIL-1 β by mouse granzyme A. [³⁵S]Methionine-labeled pIL-1 β was synthesized in a rabbit reticulo-cyte lysate system and incubated with 200 ng of Mono-S-purified ICE, granzyme B (GB), and granzyme A (GA), respectively. Enzymes were inhibited with the ICE inhibitor YVA and the general serine esterase inhibitor DCI, as indicated. (B) Western immunoblot analysis of recombinant human pIL-1 β (200 ng) incubated with GB (10 ng), GA (10 ng), and GA that had been inactivated with DCI and IGA. Incubations were conducted for 4 h at 37°C.

We next asked whether this granzyme A-generated 17-kD fragment of IL-1 β had any significant IL-1 β biological activity. We have previously shown that, in an EL-4 T helper clone, EL-4-6.1, IL-2 receptor surface expression was strictly dependent on the presence of picogram quantities of IL-1 β (19). Precursor 31 kD IL-1 β had no demonstrable activity in this assay (Table 1), as demonstrated by others previously (22). Granzyme A alone was also inactive. FACS[®] analysis revealed induction of IL-2 receptor surface expression only when pIL-1 β incubated with proteolytically active granzyme A was added. Compared to the recombinant human IL-1 β , the activity was approximately three- to fourfold lower.

pIL-1 β is synthesized as a cytosolic protein without signal peptide. Although ICE is a cytoplasmic protein, processed pIL-1 β is not observed to be associated with intact cells but is only generated and released when cells are undergoing apoptosis (23). Only when rapid apoptosis of macrophages is induced by CTLs or ATP is intracellular conversion of pIL-1 β observed (23). To evaluate whether this intracellular processing is due to granzyme A, LPS-activated macrophages were used as targets for the cytolytic T cells. The CTL clone used recognizes a peptide derived from the *P. falciparum* protein circum-

sporozoite in the context of the H2^d class I antigen. When the conversion of pIL-1 β was analyzed in macrophages during CTL attack, the anti-IL-1 β antibodies detected a faint, cell-associated band of 17 kD in addition to the 31-kD precursor, thus confirming results by Hogquist et al. (23) showing that intracellular pIL-1 β cleavage was occurring during the cytolytic attack. Processed intracellular pIL-1 β was not present in cells not previously sensitized with the peptide (Fig. 3). When CTLs were pretreated for 30 min with IGA, intracellular processing of pIL-1 β was abolished, suggesting that granzyme A may be involved in the rapid conversion of pIL-1 β (Fig. 3).

Discussion

Our results show that, although both granzyme B and ICE exhibit aspartase activity and are crucial mediators of apoptotic cell death, they do not act on identical cellular target proteins. pIL-1 β , the only known substrate for ICE, is resistant to the proteolytic action of granzyme B. Even when the ICE pIL-1 β cleavage site is offered as a 14-mer peptide, no cleavage after the critical Asp residue is observed. These observations indicate that the presence of an Asp residue alone may not be solely sufficient for the efficient processing of pIL-1 β at the Asp₁₁₆-Val₁₁₇, and other structural constraints may be required. Today no proteinaceous substrate for gran-

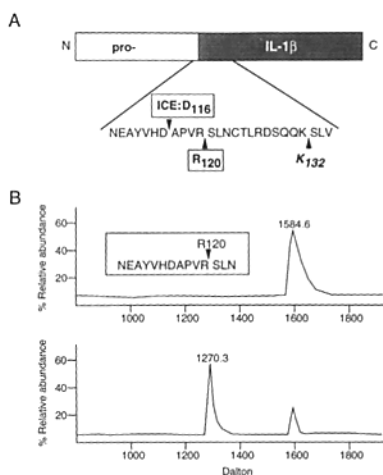


Figure 2. NH₂-terminal sequence analysis of the final cleavage product of recombinant human pIL-1 β by granzyme A. A minor cleavage site (<5%) is indicated with italic letters. Also shown is the authentic ICE cleavage site. (B) Cleavage of a synthetic peptide including the region around the cleavage site of human pIL-1 β (Asn₁₁₀ to Asn₁₂₃) by granzyme A. The cleavage site was determined by laser desorption mass spectrometry. The upper panel shows the analysis of the peptide alone. In the lower panel, the peptide had been incubated with granzyme A.

Table 1. Activities of IL-1 β

Sample	Relative activity
	%
Mature IL-1 β (17 kD)	100
pIL-1 β (31 kD)	<1
pIL-1 β (31 kD) + Granzyme A	30
pIL-1 β (31 kD) + Granzyme A + DCI	<1
pIL-1 β (31 kD) + Granzyme A + IGA	<1
pIL-1 β (31 kD) + Granzyme A	<1

IL-1 β activities were determined by the EL-4 assay described previously (19). The obtained activities were normalized to the activity of recombinant human IL-1 β , which was arbitrarily set to 100%.

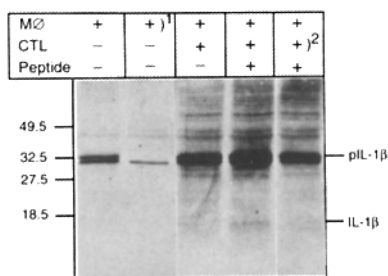


Figure 3. Intracellular processing of pIL-1 β during specific CTL-target cell interaction. Bone marrow-derived macrophages were stimulated with LPS and incubated with the CTL clone PbCSF12, which recognizes a peptide on the surface of H2^d-bearing target cells. Cells were centrifuged after a 4-h incubation and analyzed by Western blotting using an anti-mouse IL-1 β -specific antiserum. As indicated, CTLs had been pretreated with granzyme A inhibitor (IGA).² Controls include target cells that had not been pulsed with the peptide and nonstimulated macrophages.¹

zyme B has been found, in spite of its known high estero-lytic activity on synthetic thioester substrates. The absence of pIL-1 β processing by granzyme B cannot exclude, however, the possibility that other proteins, such as those activated during apoptosis, are substrates shared by ICE and granzyme B.

Unlike granzyme B, the second lymphocyte protease granzyme A expressed *in vivo* can generate an active IL-1 β from the inactive precursor form. Cleavage occurs between Arg₁₂₀ and Ser₁₂₁, yielding a cytokine four amino acids smaller in length than the one generated by ICE. Activity of the IL-1 β diminishes rapidly as the cleavage site moves away from the genuine Asp₁₁₆ cleavage site. Mast cell chymase and chymotrypsin generate molecules that are two and three residues longer with no change in activity (24). Trypsin and elastase, in contrast, generate polypeptides that are 13 and 41 amino residues longer than authentic IL-1 β but result in almost complete loss of activity (22).

The biological activity of the granzyme A-processed pIL-1 β was decreased relative to the recombinant one, in agreement with the reduction of activity observed when Arg₁₂₀ is removed or replaced by Asp (25), although structure-activity studies with truncated proteins expressed in COS cells have shown that IL-1 β retains full functional activity even when shortened to Met₁₃₆ (26). By contrast, using *in vitro*-translated IL-1 β , removal of Arg₁₂₀ resulted in decreased receptor binding (27).

Precursor IL-1 β is predominantly found in intact cells. In cells undergoing apoptosis, processing by ICE results in the release of the active cytokine, in contrast to necrosis, in which IL-1 β is released almost exclusively in the precursor form (23). Extracellular proteases such as mast cell proteases may then be expected to cleave the 31-kD precursor IL-1 β in areas of necrotic damage and thereby to release substantial amounts

of IL-1 β . Intracellular processing of pIL-1 β , however, has been observed in macrophages that are attacked by CTLs (23). CTLs cause rapid apoptosis induced by the action of granzyme B, granzyme A, and Fas ligand, a membrane protein with structural homology to TNF (15, 28, 29). Granzymes are believed to enter the target cell and act on intracellular substrates whose cleavage leads to apoptosis (30). This may account for the rapid intracellular processing of pIL-1 β . Indeed, treatment of CTLs with a granzyme A inhibitor abolished intracellular conversion of pIL-1 β in the target cell. Although the granzyme A inhibitor displays high specificity for granzyme A, it also blocks other proteases with similar specificity, such as thrombin (our own unpublished data). Whether the absence of intracellular conversion is due to the inactivation of granzyme A or to other factors remains therefore to be shown.

This lymphocyte protease-generated IL-1 β is interesting from the perspective that some cells known to synthesize pIL-1 β lack the corresponding converting enzyme (31). For example, fibroblasts and keratinocytes have been shown to produce pIL-1 β mRNA but lack IL-1 β cytokine activity (31, 32). Other secreted proteases may also play a role in processing precursor IL-1 β . Inflammatory responses of diverse origins may, in turn, be a consequence of this process. Mast cell chymase generates an active cytokine and is proposed to play a critical role in the initiation of the inflammatory response in the skin (31). Cathepsin G, which also displays pIL-1 β -converting activity, is present in synovial fluids and lung lavage from patients with inflammatory polyarthritis (32). A streptococcus-derived cysteine protease produces biologically active IL-1 β , indicating that bacteria are able to modulate the inflammatory response during pathogenesis (33). The granzyme A-generated IL-1 β may directly contribute to the inflammatory response in areas of lymphocyte tissue infiltration. IL-1 β may recruit T cells to the site of antigenic challenge caused by viruses and parasites and, in turn, activate Th lymphocytes and CTLs whose efficient activation is dependent on the IL-1 β costimulatory signal. This would lead to macrophage and neutrophil sequestration to remove cellular membrane debris caused by CTL action. In an effort to support this model, others have attempted to identify the NH₂ terminus of IL-1 β at inflammatory sites to determine the processing site, but, because of the low concentration of IL-1 β present at inflammatory sites or as a result of proteolytic degradation, they have been unsuccessful to date (32). The observation that precursor IL-1 β may be processed *in vivo* by proteases other than ICE may have important implications in the future development of therapeutic approaches. In particular, drugs currently developed to inhibit the pIL-1 β processing in monocytes by interfering with ICE activity may be unable to inhibit IL-1 β production *in vivo*.

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